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13. ABSTRACT *(Maximum 200 Words)*

Alpha-fetoprotein (AFP) is a glycoprotein produced during pregnancy by fetal yolk sac and by fetal liver and is a major protein constituent of fetal plasma throughout gestation. It has been shown that fetal physiological levels of human AFP inhibit estrogen-stimulated growth of human breast cancer. The purpose of our work is to derive a stable anti-breast cancer agent from AFP. In the past, we have identified a 14-mer peptide from the Domain III of AFP that retained the full antiestrotrophic activity AFP. In the past year, we have found that an octapeptide, P472-2 (EMTPVNPG) derived from the 14-mer peptide is the minimal sequence required to generate the antiestrotrophic activity of full-length AFP. Peptide P472-2 exhibited dose-dependent inhibition of growth of estrogen-stimulated immature mouse uterus with the optimal dose of 1 ug. P472-2 also inhibited tamoxifen-stimulated growth of immature mouse uterus. However, this activity of P472-2 was diminished during storage in the lyophilized state. Mass spectrometry analysis suggested that there were no chemical modifications during storage. Gel-filtration chromatography suggests that the peptide aggregates to form inactive species during storage. These results suggest that further chemical modification is required to generate an optimal analog with longer shelf-life.

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Jassi Mestan 7/5/00

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Table of Contents

1. INTRODUCTION

Alpha-fetoprotein (AFP) is a glycoprotein produced during pregnancy by fetal yolk sac and by fetal liver and is a major protein constituent of fetal plasma throughout gestation (1). One important biological role of AFP involves regulation of hormone-dependent responses, including estrogen-dependent growth. The Albany AFP Research Group has been studying the regulation of breast cancer growth by AFP (2-10). In those studies, full-length AFP, isolated from a variety of sources including a human hepatoma cell line (Hep G-2), stopped growth of estrogen-dependent (but not estrogen-independent) breast cancer growing as xenografts under the kidney capsule of immune deficient (SCID) mice. Part of the AFP Research Group's effort has been directed toward finding the portion of the AFP molecule responsible for its inhibition of estrogen-stimulated cancer growth. Molecular biology tools were used to parse the AFP molecule into its domains and subdomains (8) and synthetic peptides from active subdomains were generated using solid phase peptide synthesis. These products were tested in our anti-estrotrophic screening assay which measures inhibition of estrogen-stimulated growth of immature mouse uterus. As shown in Table 1 below, the antiestrotrphic activity is localized in a 14-mer peptide (amino acids 467-480).

TABLE 1. Activity of AFP-Derived Agents in Immature Mouse Uterine Growth Assay

TEST AGENT	DOSE*	INHIBITION OF GROWTH
Full-length Natural Human AFP	(100 μg)*	41**
Full-length Recombinant Human AFP	(100 μg)	34**
Recombinant Domain I ABC AFP	(10 μg)	0
Recombinant Domain III ABC AFP	(10 μg)	24
Recombinant Domain III AB AFP	(10 μg)	32**
AFP Synthetic Peptide 428 (amino acids 428-444)	(1 μg)	5
AFP Synthetic Peptide 447 (amino acids 447-480)	(1 μg)	36**
AFP Synthetic Peptide 511 (amino acids 511-580)	(1 μg)	0
AFP Synthetic Peptide 447-4 (amino acids 447-456)	(1 μg)	10
AFP Synthetic Peptide 457 (amino acids 457-466)	(1 μg)	0
AFP Synthetic Peptide 467 (amino acids 467-480)	(1 μg)	42**
Full-length Natural Albumin	(100 μg)	6
Albumin Synthetic Peptide (amino acid 440-473)	(1 μg)	2
Tamoxifen	(10 μg)	0

* The optimal inhibitory dose per mouse given by the i.p. route is shown in parentheses for each agent. The optimal stimulatory dose of estrogen (0.5 μg per mouse) was given by the i.p. route one hour after injection of test agent. Twenty-two hours later uteri were dissected, weighed, and compared to control groups which received no estrogen (negative control) or estrogen alone (positive control).
** Significant inhibition, $p<0.05$; Wilcoxon Sum of Rank Test

These exciting findings suggested that the long road from intact AFP, to Domain III, to Subdomain IIIAB, to 34-mer peptide P447, to 14-mer peptide P467 was successful in identifying the active site of the native anti-oncotic protein. However, subsequent experiments showed that peptides P447 and P467 aggregate upon storage, accompanied by loss of activity. We had hypothesized that we could develop an even better, smaller, and more stable analog. During the first year of this training grant, we were able to identify an octapeptide as the minimal active sequence that retains full activity (see the attached manuscript and Figure 1).

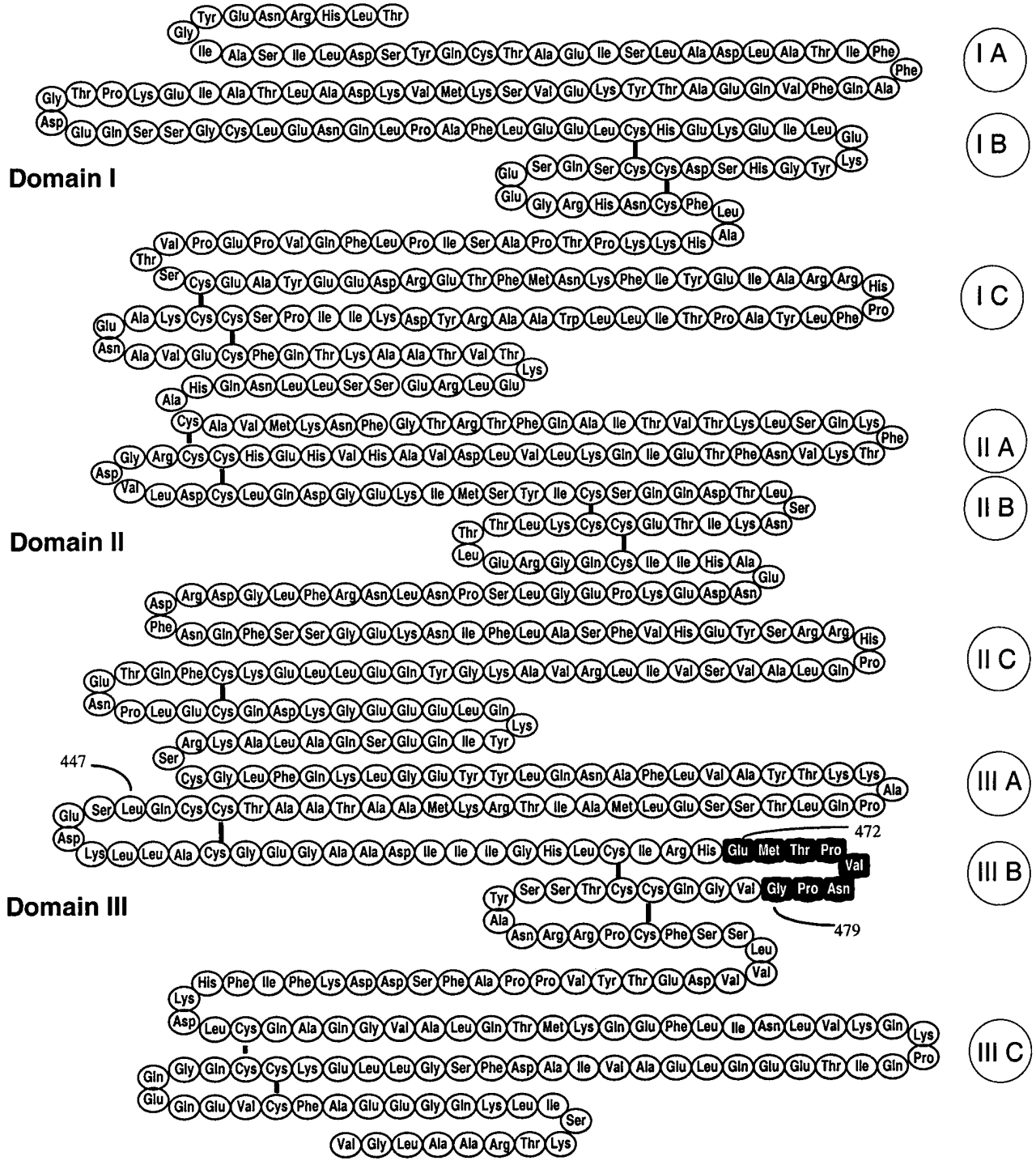


Figure 1. Amino acid sequence, domain and subdomain designation and disulfide bonding pattern of human AFP. Peptide 472-2 is designated by darkened ovals.

2. BODY

2.1 Training

During the first year of this training grant award, Mr. Fassil Mesfin, the recipient of the training grant, has completed a number of tasks that are required for award of the Ph.D. degree. Mr. Mesfin completed the didactic courses and passed the written and oral qualifying examinations. He has one first-authorship manuscript, two abstracts, and one co-authorship manuscript. Mr. Mesfin performed six intramural and two extramural research presentations. The intramural presentations were performed during the doctoral thesis committee meetings, weekly department seminars, the annual research retreat meeting, and the annual graduate studies program research day. The extramural presentations were performed at the 1999 Steroid Receptor Superfamily Special Conference and at the 2000 annual meeting of the American Association for Cancer Research (AACR). In 1998 and 1999, Mr. Mesfin was named an AACR Minority Scholar in Cancer Research. Additionally, he was the recipient of the 1999 Dean's Prize for Excellence in Extramural Research Activities, the 2000 Dean's Prize for Excellence in Research, and the 2000 Richard H. Edmonds Alumni Award for outstanding student leadership from Albany Medical College. Mr. Mesfin has met twice with his thesis examination committee. Dr. James A. Bennett (Pharmacology, Surgery, and Cancer Therapeutics) is mentor and the Chair of the thesis committee. Dr. Thomas T. Andersen (Protein Chemistry), Dr. Herbert I. Jacobson (Endocrinology), Dr. Bruce R. Line (Radiology and Diagnostic Imaging), Dr. John Gierthy (Cell Biology), and Dr. Susan Baxter (NMR Spectroscopy) are the voting members of the thesis committee.

2.2. Research

2.2.1. Methods

During the first year of this training grant the student has spent significant amount of time in peptide synthesis and characterization. All peptides were synthesized by using Fmoc solid phase peptide synthesis method. The peptides were purified using reverse phase high pressure liquid chromatography. The amino acid composition of the peptides was confirmed by Amino Acid analysis. The molecular weight of the peptides was determined by mass spectrometry. Possible secondary structure of the peptides was analyzed using internet-based programs and circular dichroism spectroscopy. There were no NMR studies performed at this stage of the training because we do not have the optimal analog. The aggregation state of the peptides were assessed by gel-filtration chromatography. The octapeptide analogs seem to show a very low resolution of aggregates and further work is needed to resolve this problem. The biological activity of the peptides were determined by estrogen-stimulated immature mouse uterine growth assay, T47 human breast cancer cell proliferation assay, and MCF7 human breast cancer xenograft growth assay.

2.2.2. Results

I. Production of 14-mer peptide P467 analogs.

Two 10-mer peptides (447-456, 457-466) and one 14-mer peptide (467-480) were synthesized to identify the active site of 34-mer peptide P447. . The two 10-mer peptides were derived from the amino terminal and middle of 34-mer peptide P447, while a 14-mer peptide P467 was derived from the carboxyl terminus of 34-mer peptide P447. The 14-mer peptide P467

exhibited significant antiestrotrophic activity while the 10-mer peptides had no antiestrotrophic activity (Table 1).

Peptide P467 is less than half of the size of the 34-mer peptide P447 but it retained the full biological activity (Table 1). Like P447, the biological activity of P467 was diminished during storage in lyophilized state and the diminished activity of these peptides was recovered by 4 M urea treatment (Table 2). From this we hypothesized that the peptides formed aggregates during storage. Gel-filtration chromatography (GFC) analysis of the 34-mer peptides confirmed that the peptides aggregate to form inactive species (Figure 2). Because of the molecular weight limitation of GFC, aggregates of the 14-mer peptides and smaller analogs have not yet been resolvable by GFC.

TABLE 2. Activity of AFP-Derived 34-mer and 14-mer Peptides During Storage

NAME	STORAGE TIME (weeks)	INHIBITION OF GROWTH
P447 (34-mer)	0	40**
P447 (34-mer)	3 or more	5
P447 (34-mer) HMW	3 or more	0
P447 (34-mer) LMW	3 or more	34**
P447 (34-mer) + Urea	3 or more	29**
P467 (14-mer)	0	41**
P467 (14-mer)	4 or more	10
P467 (14-mer) + Urea	4 or more	35**

The optimal inhibitory dose (1 μg peptide per mous) given by the i.p. route is shown in parentheses for each agent. The optimal stimulatory dose of estrogen (0.5 μg per mouse) was given by the i.p. route one hour after injection of test agent. Twenty-two hours later uteri were dissected, weighed, and compared to control groups which received no estrogen (negative control) or estrogen alone (positive control). HMW is high molecular weight aggregate (>10,000) and LMW is low molecular weight fraction (<10,000). HMW and LMW samples were prepared using GFC.

** Significant inhibition, $p<0.05$; Wilcoxon Sum of Rank Test.

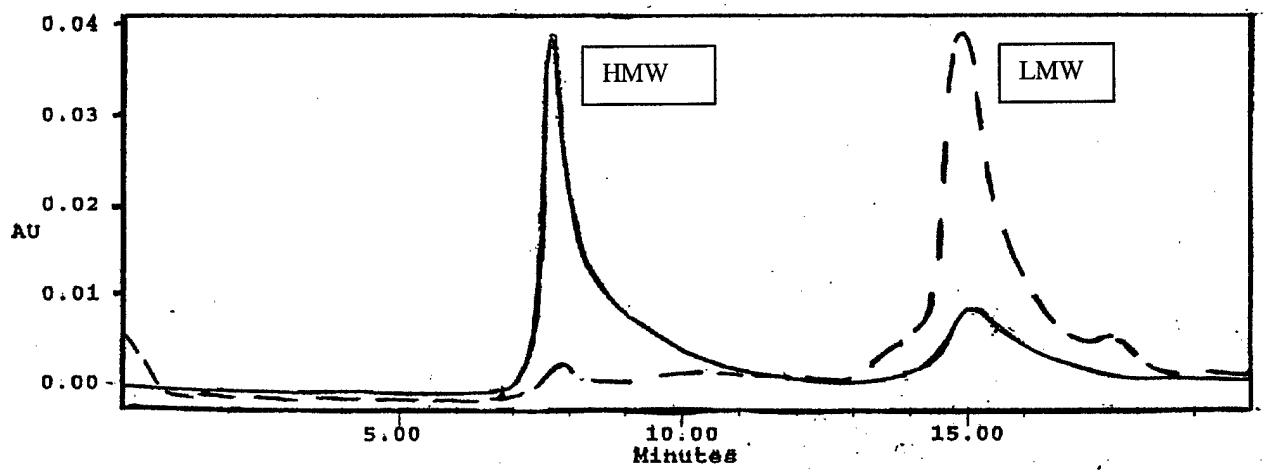


Figure 2. Gel-Filtration Chromatography Profile of the Stored 34-mer Peptide P447. ___ Profile of stored P447 (after 1 or more years of storage). ---- Profile of stored peptide after urea treatment.

We have generated peptide P467 analogs in an attempt to prevent aggregation that was associated with the loss of biological activity (Table 3). P467-biotin was biotinylated at the cystine residue to prevent inter-disulfide bond formation that might lead to aggregation. P467-1 was an alanine substituted analog also that was expected to reduce aggregation by preventing inter-disulfide bond formation. P467-2 was aspartic acid substituted analog that was expected to

reduce aggregation by preventing inter-disulfide bond formation and increasing the hydrophilicity of the peptide. P467-3 was triple aspartic acid substituted analog, which was very hygroscopic and expected to improve the solubility of the peptide.

TABLE 3. Activity of AFP-Derived 14-mer Peptide Analogs

NAME	PEPTIDE SEQUENCE	INHIBITION OF GROWTH
P467	LCIRH EMTPV NPGV	41**
P467-biotin	LCIRH EMTPV NPGV (biotinylated cystine)	34**
P467-1	LAIRH EMTPV NPGV	44**
P467-2	LDIRH EMTPV NPGV	40**
P467-3	**DDD**RH EMTPV NPGV	30**

The optimal inhibitory dose (1 μg peptide per mous) given by the i.p. route is shown in parentheses for each agent. The optimal stimulatory dose of estrogen (0.5 μg per mouse) was given by the i.p. route one hour after injection of test agent. Twenty-two hours later uteri were dissected, weighed, and compared to control groups which received no estrogen (negative control) or estrogen alone (positive control).
** Significant inhibition, $p<0.05$; Wilcoxon Sum of Rank Test

Fresh preparation of these analogs showed significant biological activity in the immature mouse growth uterine growth assay. However, like the native peptide, the activities of these analogs were diminished during storage in the lyophilized state. Since chemical modification and amino acids substitution at the N-terminal residues did not affect the biological activity, we hypothesized that the activity of the 14-mer peptide was localized at the C-terminal residues.

II. Determination of the Minimal Active Sequence

To identify the minimal sequence required for antiestrotrophic activity, truncated forms of the 14-mer, 467-1, were synthesized. Analogs were a 10-mer peptide (P471), a 9-mer peptide (P472-1), an 8-mer peptide (P472-2), three 7-mer peptides (P472-3, P473, P474), and a 5-mer peptide (P472-4). All the peptides in this category were derived from the carboxyl terminus of 14-mer peptide P467-1 (Table 2).

Peptide P472-2 is an 8-mer peptide from the C-terminus of the 14-mer, and this small peptide exhibited significant antiestrotrophic activity (Table 2). However, peptides smaller than the 8-mer, P472-3 (7-mer missing the 8-mer C-terminal residue), P473 (7-mer missing N-terminal residue of the 8-mer), P474 (7-mer missing two N-terminal residues of the 8-mer and having an additional C-terminal residue), and P472-1 (5-mer peptide missing three C-terminal residues of the 8-mer) were all without significant inhibitory activity (Table 4).

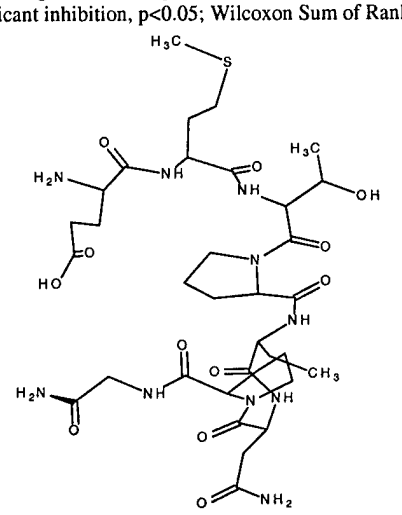
TABLE 4. Activity of 14-mer Peptide-Derived Truncated Analogs

Name	**Peptide Sequence**	**% Growth Inhibition**
P467-1	LAIRH EMTPV NPGV	40**
P471	H EMTPV NPGV	38**
P472-1	EMTPV NPGV	40**
P472-2	EMTPV NPG	49**
P472-3	EMTPVNP	20
P472-4	EMTPV	5
P473	MTPV NPG	0
P474	TPV NPGV	0

TABLE 5. AFP-Derived Octapeptide P472-2 Inhibits Mouse Uterine Growth Stimulated by Estradiol (E2) or Tamoxifen (Tam).

Injectant 1	Injectant 2	Mean Uterine Weight ± SE	% Growth Inhibition
Saline	Saline	1.06 ± 0.07	
Saline	E2	1.85 ± 0.08	
AFP	E2	1.55 ± 0.03	38**
P472-2	E2	1.49 ± 0.06	46**
Scrambled P472-2	E2	1.82 ± 0.05	4
Saline	Tam	2.68 ± 0.06	
P472-2	Tam	2.24 ± 0.10	27*
Saline	P472-2	0.98 ± 0.02	

The optimal inhibitory dose (1 μg peptide per mouse) given by the i.p. route is shown in parentheses for each agent. The optimal stimulatory dose of estrogen (0.5 μg per mouse) was given by the i.p. route one hour after injection of test agent. Twenty-two hours later uteri were dissected, weighed, and compared to control groups which received no estrogen (negative control) or estrogen alone (positive control).
** Significant inhibition, $p<0.05$; Wilcoxon Sum of Rank Test



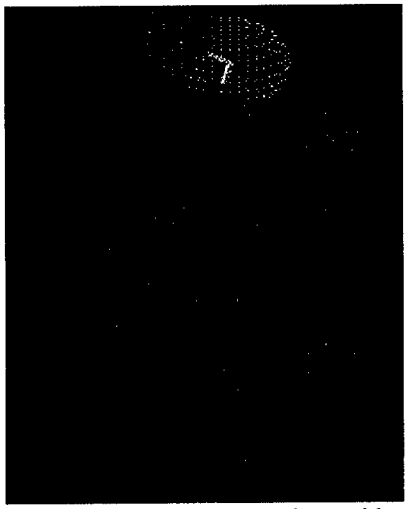
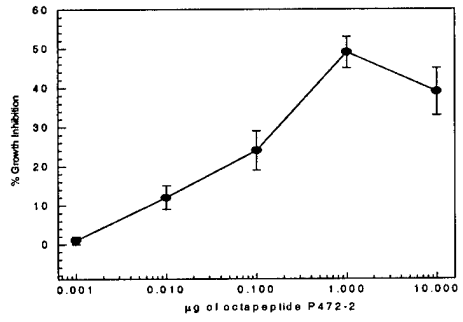


Figure 3. Energy minimized structure of octapeptide P472-2. The energy minimization calculations were done with ChemSketch program based on CHARMM parameterization.



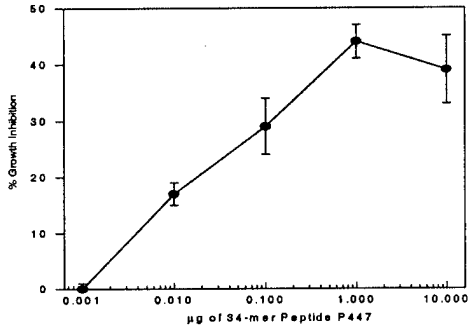


Figure 4. Dose-dependent antiestrotrophic activity of AFP-derived octapeptide P472-2 and 34-mer peptide P447. These results demonstrate that both peptides significantly inhibited estrogen-stimulated growth of immature mouse uterus with a similar dose/response profile and an optimal dose of 1 ug per mouse.

These results indicated that the 8-mer peptide (amino acids 472-479 of AFP) is the minimal sequence from AFP that retains the antiestrotrophic activity of the full length molecule. A dose

response curve of this peptide is shown in Figure 4. It is virtually superimposeable on the dose response curve of the 34-mer peptide P447 suggesting no loss of activity as a result of truncating the larger peptide. Tamoxifen stimulates the growth of the uterus, but the octapeptide (alone) does not stimulate the growth of the uterus (Table 5). In fact, the peptide inhibits tamoxifen-stimulated uterine growth (Table 5). This brings to light two potential uses for the peptide: it may serve in combination therapy with tamoxifen to help prevent the adverse uterotrophic effect of tamoxifen, and it may be useful in the treatment of tamoxifen-resistant breast cancers.

III. Anti-Breast Cancer Activity of AFP-Derived Octapeptide P472-2

The anti-breast cancer activity of the octapeptide was assessed against estradiol-stimulated growth of T47D human breast cancer cells in culture. As shown in Figure 3, AFP-derived octapeptide inhibited T47D growth by 65% (similar to 34-mer peptide P447, data not shown). Raloxifene (LY 156758) produced almost 100% growth inhibition of these cells in culture. Additionally, AFP-derived peptide analogs inhibited estrogen-dependent MCF7 human breast cancer xenograft growth.

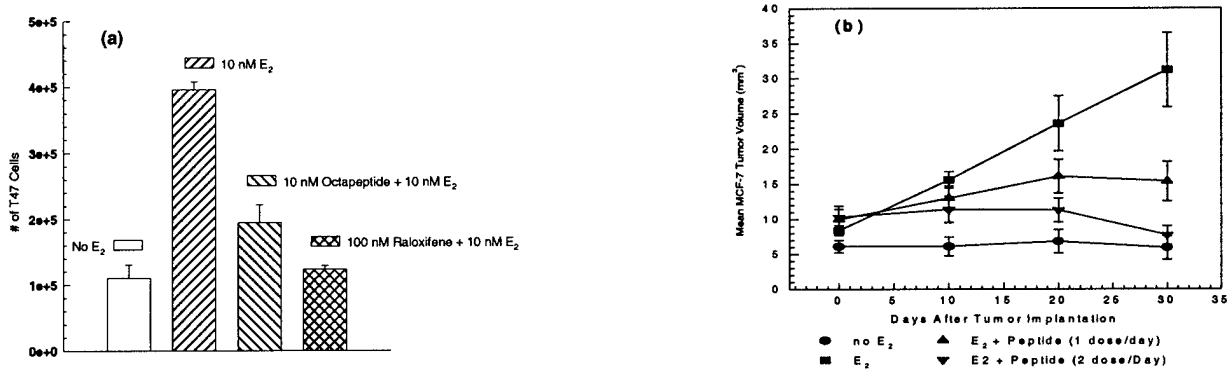


Figure 5. Growth inhibitory activity of AFP-derived peptide against (a) estrogen-dependent T47D human breast cancer cells growing in culture and (b) estrogen-dependent MCF7 human breast cancer cells growing as xenografts. These results show that AFP-derived peptide inhibited estrogen-stimulated growth of human breast cancer cells in culture and in vivo.

3. DISSCUSION

The synthetic nature and defined structure of this octapeptide suggest that it can be developed into a new drug that opposes the action of estrogen, perhaps including the promotional effects of estradiol in the development of human breast cancer. Further, preliminary data from our research group suggests that the octapeptide works by a mechanism other than that of tamoxifen, and so it may be useful in an adjunctive role with tamoxifen or as a replacement for tamoxifen in those cancers which are resistant to tamoxifen.

The data in this report demonstrate that an octapeptide, P472-2 (amino acids 472-479 of AFP), possessed the entire antiestrotrophic activity contained in full-length AFP. Festin et al. [8] had previously shown that this activity was contained in the third domain of AFP (amino acids

sequence 386-592), and Mizejewski et al [11] showed the activity resided in a 34-mer peptide (amino acid 447-480) from Domain III of AFP. This 8-mer peptide is a substantially truncated form of the 34-mer and yet retains significant activity. Smaller regions within this 8-mer were without activity. A homology search revealed a five-amino acid sequence (EMTPV, amino acids 472-476 of AFP) that was found in the tumor suppressor protein E-cadherin. This 5-mer peptide was therefore synthesized and tested, and it showed no significant antiestrotrophic activity. The AFP-appropriate amino acids were added to the carboxyl end of this peptide and no significant activity was obtained until the 8-mer (amino acids 472-479 of AFP) was reached. Thus the 8-mer is the minimal sequence required for antiestrotrophic activity, and this appears to be the active site of AFP that imparts this activity to the full-length protein.

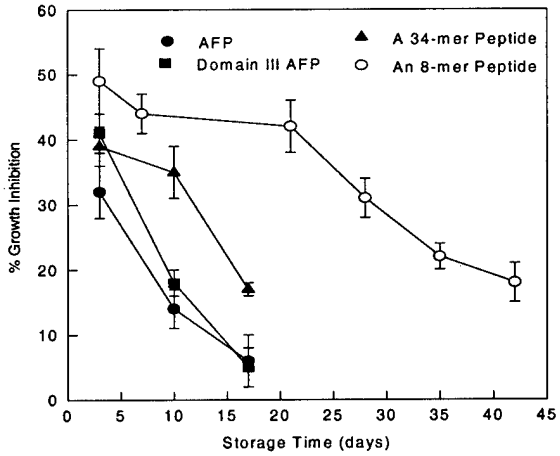


Figure 6. The antiestrotrophic activity of AFP and Fragments of AFP following storage. These results show that the activity of the full-length AFP and its active fragments was diminished during storage. However, the smallest active sequence, octapeptide P472-2 exhibited better shelf-life.

Full-length AFP, Domain III AFP and the AFP-derived peptides described herein lose activity during storage (Figure 6). However, the 8-mer did retain its activity longer than the other analogs during storage. Wu et al. [12] reported that AFP tends to form aggregates which may contribute to its loss of activity. Lai et al. reported that peptides are prone to chemical degradation pathways even upon storage in the solid state [13]. Possible chemical modifications of the 8-mer peptide P472-2 might include covalent modifications such as deamination of asparagine, oxidation of methionine, or N-terminal pyroglutamate formation, each of which could be detected by mass spectrometry analysis. Mass spectrometry analysis of the 8-mer peptide P472-2 when freshly prepared, or after storage in the solid state for over three months, indicated the expected molecular weight of 842. This result suggested that the 8-mer peptide P472-2 was free of these chemical modifications and that chemical modification was not the cause of activity loss during storage. In addition to chemical instability, peptides may be prone to physical instability that alters their biological activity [14]. It was noted from gel filtration chromatography profiles that the 34-mer peptide P447 (the parent peptide of P472-2) would

readily form dimers, trimers, and large molecular weight aggregates (unpublished observation). This aggregation process of the 34-mer peptide in the lyophilized state resulted in a time-dependent loss of biological activity as assessed by the immature mouse uterine growth bioassay. Preliminary studies suggest that the 8-mer, like the 34-mer, forms aggregates during storage in the lyophilized state. Therefore, a further chemical modification of the peptide is necessary to prevent aggregation and concomitant loss of activity during storage.

This AFP-derived octapeptide, if it behaves like full-length AFP, will be a novel agent in that it is not cytotoxic like most standard cancer chemotherapeutic agents. It is not an estrogen receptor antagonist like tamoxifen (unpublished data). It will not deplete estrogen levels like inhibitors of estrogen synthesis (4-hydroxyandrostenedione). It will not bind and sequester estrogen (15). It is highly likely that we have discovered an agent that is anti-oncotic through a novel mechanism. This will open up new therapeutic possibilities for not only this agent, but also other agents which may interfere with the putatively unique mechanistic pathway. We are still in the developmental stages of this peptide, but we are confident that the results of this study will place us in a much better position to propose preclinical and clinical trials of AFP-derived peptide as a novel therapeutic agent for breast cancer.

4. FUTURE WORK

For the next year, the recipient of this training award will spend most of the time on refinement of octapeptide. We will optimize our gel filtration chromatography system so that we can characterize the aggregation state of the octapeptide. Then we will make modifications to the existing octapeptide to make it even better in terms of potency and storage characteristics (shelf- life). We believe that increasing the hydrophilicity of the peptide might prevent the aggregation and loss of biological activity. So, we will design and generate a number of hydrophilic octapeptide analogs. We will also generate structurally restricted peptide analogs by cyclization. Cyclization might increase the in vivo half-life of the analogs by minimizing the degradation of the analogs by exopeptidases. Further, we will generate combinatorial and D-amino acid substituted analogs and characterize the biological activity in both cell culture and in vivo.

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6. APPENDICIES

1) Research Accomplishments

- AFP-derived peptides aggregate during storage in lyophilized state.
- AFP-derived Octapeptide is the minimal sequence required for the antiestrotrophic activity of full-length AFP.
- The Octapeptide inhibited the growth of estrogen-dependent human breast cancer in cell culture and in vivo.
- Like AFP and AFP-derived agents, the activity of the octapeptide diminished during storage in lyophilized state.

2) Reportable Outcomes

Manuscripts

Mesfin FB, Bennett JA, Jacobson HI, Zhu S, and Andersen TT, Alpha-fetoprotein-derived antiestrotrophic octapeptide, Biochimica et Biophysica Acta 1501 (2000) 33-43

Eisele LE, Bennett JA, Mesfin FB, Andersen TT, Jacobson HI, Soldwedel H, MacColl R, and Mizejewski GJ. Aggregation Behavior of a Synthetic Biologically-Active peptide Derived from Alpha-Fetoprotein. (Submitted May 2000)

Abstracts and Presentations

Fassil B. Mesfin, Thomas T. Andersen, James A. Bennett, ShuJi Zhu, Herbert I. Jacobson. Alpha-Fetoprotein-Derived Anti-Estrogenic Peptides., American Association for cancer Research Special Conference, The Steroid Receptor Superfamily, Palm Spring, CA January, 1999.

Herbert I. Jacobson, Thomas T. Andersen, Gerald J. Mizejewski, George Butterstein, Fassil B. Mefin, ShuJi Zhu, and James A. Bennett, Alpha-Fetoprotein Inhibits Cellular Response to Estrogen, Androgen, Glucocorticoid, and Thyroid Hormone. Is it a Superfamily Inhibitor? American Association for Cancer Research Special Conference, The Steroid Receptor Superfamily, Palm Spring, CA, January 1999.

Degrees obtained that are supported by this award

Albany Medical College awarded Master of Science Degree in Biomedical Science to Fassil Mesfin in May 2000.

3) Original and two copies of each of the above cited manuscripts and abstracts.

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Alpha-fetoprotein-derived antiestrotrophic octapeptide

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Abstract

Alpha-fetoprotein (AFP) is a major serum protein produced during fetal development. Experimental findings suggest that AFP has antiestrotrophic activity and that it can be developed as a therapeutic agent to treat existing estrogen-dependent breast cancer or to prevent premalignant foci from developing into breast cancer. The antiestrotrophic activity of AFP was reported to be localized to a peptide consisting of amino acids 447–480, a 34-mer peptide termed P447. A series of parsings and substitutions of amino acids in the P447 sequence was intended to identify the shortest analog which retained antiestrotrophic activity. Peptides related to P447 were generated using solid phase peptide synthesis. Several shorter peptides, including an 8-mer called P472-2 (amino acids 472–479, peptide sequence EMTPVNPG), retained activity, whereas peptides shorter than eight amino acid residues were inactive. The dose-related antiestrotrophic activity of AFP-derived peptides was determined in an immature mouse uterine growth assay that measures their ability to inhibit estradiol-stimulated uterine growth. In this assay, the maximal inhibitory activities exhibited by peptide P472-2 (49%), by peptide P447 (45%), and by intact AFP (35–45%) were comparable. The octapeptide P472-2 was also active against estradiol-stimulated growth of T47D human breast cancer cells in culture. These data suggest that peptide P472-2 is the minimal sequence in AFP, which retains the antiestrotrophic activity found with the full-length molecule. The synthetic nature and defined structure of this 8-mer peptide suggest that it can be developed into a new drug which opposes the action of estrogen, perhaps including the promotional effects of estradiol in the development of human breast cancer.

Keywords: Alpha-fetoprotein; Synthetic octapeptide; Breast cancer

1. Introduction

Alpha-fetoprotein (AFP) is a glycoprotein produced during pregnancy by the fetal yolk sac and by fetal liver and is a major protein constituent of fetal plasma throughout gestation [1]. It has a molecular weight of approximately 69 kDa and has 39% primary structural homology to albumin [2]. It has been proposed that the tertiary structure of AFP is composed of three domains based on its disulfide bonding pattern [3]. In studies designed to assess the regulation of breast cancer growth by AFP [4–13], we have reported that full-length AFP, isolated from a variety of sources including a human hepato-

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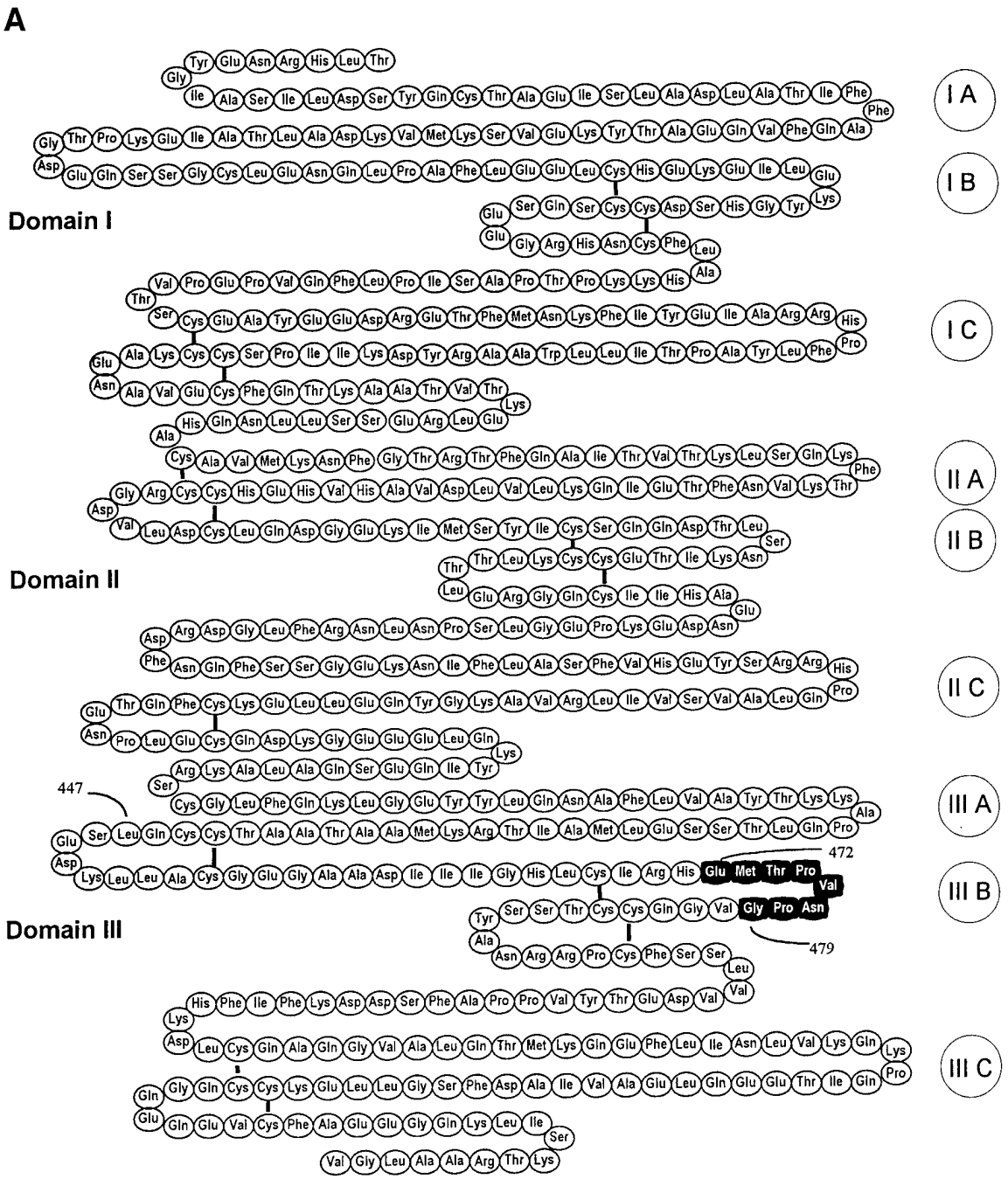

Fig. 1. (A) The primary structure, domain and subdomain designation of AFP. Black circles represent the 8-mer peptide P472-2. The 8-mer peptide is the minimal sequence required for antiestrotrophic activity. (B) Energy-minimized structure of peptide P472-2. Amino acids are labeled using three-letter code. The energy minimization calculations were done with ChemSketch program based on CHARMM parameterization.

ma cell line (HepG-2), stopped growth of estrogen-dependent breast cancers, but did not inhibit the growth of estrogen-independent breast cancers when these were growing as xenografts under the kidney capsule of immune-deficient (SCID) mice [4].

Part of our effort has been directed toward finding the active site of the AFP molecule that is responsi-

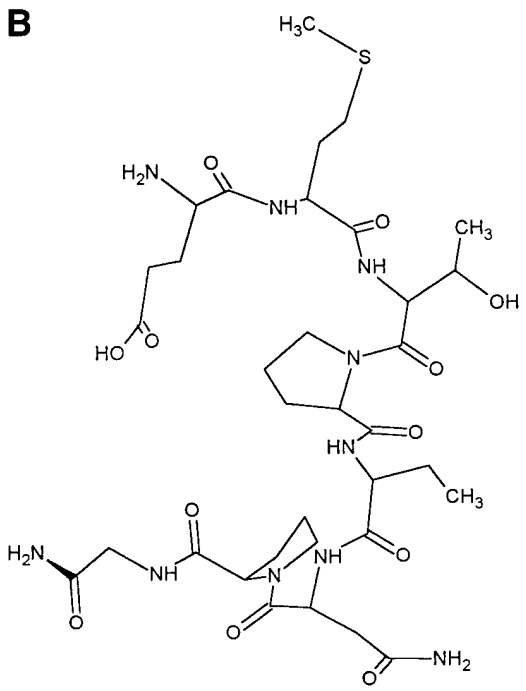

Fig. 1 (*continued*).

ble for its ability to inhibit the growth of estrogen-stimulated breast cancer. Molecular biology tools were used to parse the AFP molecule into its domains and subdomains (Fig. 1A). Domain III and subdomain IIIAB were found to be active [10,11]. Synthetic peptides from the active subdomain of domain III were generated using solid phase peptide synthesis, and a 34-mer peptide (amino acids 447–480) was found to be active [14]. In this paper, we report that an AFP-derived octapeptide comprised of amino acids 472–479 (Fig. 1) arising from the carboxy-terminus of the above 34-mer peptide (amino acids 447–480) is the minimal sequence required for antiestrotrophic activity (Fig. 1).

2. Materials and methods

2.1. Secondary structure prediction and homology search

Potential secondary structures of peptide P447 were predicted using Predictprotein, an internet-based program which reportedly predicts secondary structure of proteins and solvent accessibility at better than 70% accuracy [15]. In addition, a sequence homology assessment was performed using the protein data bank of Research Collaboratory for Structural Bioinformatics. Energy minimization computations were done with ChemSketch program. The program produces energy-minimized structure based on CHARMM parameterization [16]. Note that the program is not a full-scale molecular mechanics engine. Its design aims were to reliably reproduce reasonable conformations of 3D structure.

2.2. Peptide synthesis

Peptides were synthesized using Fmoc solid phase peptide synthesis on a Pioneer Peptide Synthesis System (PerSeptive Biosystem, Inc.). Briefly, peptides were assembled on a solid support (Fmoc–polyethylene–graft polystyrene support) from the C-terminus, reacting the deblocked N-terminus of support-bound amino acid with the activated C-terminus of the incoming amino acid to form an amide bond. Amino acids used in the synthesis had their N^{α}-amino group protected by the 9-fluorenylmethoxycarbonyl (Fmoc) group, which was removed by piperidine at the end of each cycle in the synthesis. Side-chain protecting groups of amino acids were Arg(Pbf), Asn(Trt), Asp(OtBu), Gln(Trt), Glu(OtBu), His(Trt), Lys(tBoc), Ser(tBu), Thr(tBu), Cys(Trt), which were deprotected by trifluoroacetic acid (TFA) after peptide synthesis. The carboxyl group of the amino acid was activated with HATU [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] obtained from PerSeptive Biosystems Inc. The specific amino acid derivatives, supports, and reagents used in the synthesis were purchased from PerSeptive Biosystems Inc., Framingham, MA, USA.

Peptides were cleaved from the solid support (polyethylene–graft polystyrene) using TFA. After synthesis was completed, the resin was washed three times with 100% methanol and the cleavage reaction was initiated by incubating the resin in 10 ml TFA/thioanisole/anisole/1,2-ethanedithiol (90:5:2:3) per 0.5 g resin for 5 h (or other appropriate conditions). The cleavage reaction mixture was filtered using a sintered glass funnel to separate the solid resin from the peptide solution. Filtrate volume was reduced to 1 ml with a gentle stream of air and the peptides were precipitated by addition of 15 ml dry-

ice chilled ethyl ether. The peptides were allowed to settle for 5 min at −80°C, and the supernatant was aspirated. The peptides were then washed twice in similar manner with 15 ml of ethyl ether. After three further washes with 15 ml of 1.5:1 ethyl acetate: diethylether (room temperature), the peptides were dissolved in deionized water and lyophilized.

2.3. Purification of peptides

Lyophilized peptides were dissolved in deionized water and subjected to reversed-phase chromatography with C18 Sep-Pak Cartridges (Waters Inc.), or to gel filtration on Sephadex G-25. Subsequently, peptides were lyophilized, dissolved in deionized water and further purified using analytical or semi-preparative reversed phase HPLC.

2.4. Peptide characterization

Amino acid analysis of all peptides was performed using the Waters AccQ-Tag amino acid analysis system [17,18]. Additionally, peptides were analyzed by mass spectrometry using standard α-cyano-4-hydroxysinnipinic acid and sinnipinic acid matrices.

2.5. Immature mouse uterine growth assay

A bioassay for antiestrotrophic activity was performed according to Mizejewski et al. [19]. Swiss/Webster female mice, 6–8 g in body weight (13–15 days old), were obtained from Taconic Farms (Germantown, NY). Mice were weighed and distributed into treatment groups (typically five mice per group) such that each group contained the same range of body weight. In a typical experiment, each group received two sequential intraperitoneal injections 1 h apart. Test material or vehicle control for that material was contained in the first injectant. Estrogen or vehicle control for estrogen was contained in the second injectant. Twenty-two hours after the second injection, uteri were dissected, trimmed free of mesenteries, and immediately weighed. The uterine weights were normalized to mouse body weights (mg uterine weight/g of body weight) to compensate for differences in body weight among litters of the same age. Experiments employed a minimum of five mice per group, and the mean normalized uterine weights and standard error for each group were calculated. Percent growth inhibition in a test group was calculated from the normalized uterine wet weights according to Eq. 1. Groups were considered to be significantly different at $0.05 \geq P$, employing the non-parametric one- sided Wilcoxon sum of ranks test.

$$\text{Growth inhibition (\%)} = \frac{\text{Full } E_2 \text{ stimulation} - E_2 \text{ stimulation in test group}}{\text{Full } E_2 \text{ stimulation} - \text{No } E_2 \text{ stimulation}} \times 100 \quad (1)$$

2.6. T47 cell proliferation assay

A confluent culture of T47D human breast cancer cells growing in culture was released from monolayer by trypsinization (0.25% trypsin and 0.25% EDTA). Stock T47D cells were suspended in phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% dextran/charcoal-treated fetal bovine serum (DCFBS). Cells were then seeded into 6 well plastic tissue culture plates at a density of 1×10^5 cells/well in 4 ml of medium. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The cells were depleted of steroids by replenishment of DCFBS-supplemented medium every other day for 5 days. After steroid depletion, estradiol (10 nM E_2), vehicle, or E_2 with various concentrations of test agents were added with medium change every other day for 9 days. At the end of each experiment, cells were released from monolayer by trypsinization, diluted in a 1:1 ratio of 0.1% trypan blue, and placed in a hemocytometer and counted. Wells were set up in triplicate for each group. Mean viable cell numbers and the standard error were calculated for each group.

3. Results

The 34-mer AFP-derived peptide described by Mizejewski et al. [14] was synthesized. It contained amino acids 447–480 of AFP (Fig. 1). Mass spectrometry analysis showed the expected molecular weight of the peptide and amino acid analysis re-

vealed that it had the expected amino acid composition. As shown in Fig. 2, it significantly inhibited estrogen-stimulated growth of immature mouse uterus, and its optimal dose was 1.0 μg per mouse.

Potential secondary structures of the 34-mer AFP-derived peptide (amino acids 447–480 in AFP) were assessed using Predictprotein [15], which suggested that the amino acids in sequence positions 461–471 of peptide P447 have potential to form extended β-sheet structure, while the amino- and the carboxy-termini have potential to form a random coil structure. Predictprotein predicted no potential for α-helix structure. Peptide P447 has two cysteine residues (C455, C468), which have the potential to form intra- or inter-peptide disulfide bonds. These observations led us to produce a number of P447 analogs with amino acid substitutions designed to alter or eliminate the potential β-sheet structures or to prevent disulfide formation (Table 1).

The specific rationales for designing analogs of P447 were:

1. To prevent disulfide bond formation. Analogs in this category included 34-mer peptides (P447-1, P447-2, P447-3), in which substitutions for the two cysteines were made (Table 1).
2. To reduce the β-sheet content. Analogs in this category include two 34-mer peptides (P447-2, P447-3), in which cysteine was substituted with aspartate or proline, respectively (Table 1). Aspartate substitution was expected to reduce potential β-sheet structure formation and to increase the solubility of the peptide, while proline substitution was expected to reduce potential β-sheet structure formation and increase random coil structure. These substitutions also eliminated the possibility of disulfide bond formation as did substitution with alanine to produce analog P447-1.
Biological activities of the peptides were evaluated using the immature mouse uterine growth bioassay as described in Section 2. AFP-derived 34-mer peptides P447, P447-1, P447-2, and P447-3 exhibited significant inhibitory activity (Table 1). However, their activities were reduced after storage for 3–4 weeks (data not shown). Importantly, P447-1 is active and contains no cystine or cysteine. Those residues are therefore not essential for antiestrotrophic activity, and disulfide bond formation is evidently not the cause of loss of that activity during storage. In addition, substitution that would disrupt possible β-sheet structure in peptides P447-2 and P447-3 did not abolish the biological activity, which suggests that β-sheet structure might not be essential for activity. Circular dichroism is not especially revealing for peptides as small as these, undoubtedly because they can access so many different conformations in solution. Similarly, secondary structure analyses of peptides this small are usually unrevealing for the same reason, and this is the case with these peptides. Further, any relationship between the solution conformation(s) of the peptides and the conformation they may adopt when bound to the receptor is unknown. Nevertheless, these results suggested that the antiestrotrophic activity might be retained by smaller analogs of the 34-mer peptides.
3. Truncated forms of the 34-mer were synthesized

Table 1
Antiestrotrophic activity and sequence of AFP-derived 34-mer peptides

Test agent	% Inhibition of E_2-stimulated growth of immature mouse uterus	Peptide sequence
Peptide P447	441*	LSEDK LLACG EGAAD IIIGH LCIRH EMTPV NPGV
Peptide P447-1	39*	LSEDK LLA**A**G EGAAD IIIGH L**A**IRH EMTPV NPGV
Peptide P447-2	45*	LSEDK LLA**D**G EGAAD IIIGH L**D**IRH EMTPV NPGV
Peptide P447-3	30*	LSEDK LLA**P**G EGAAD I**P**IGH L**P**IRH EMTPV NPGV

Peptide 447 is an AFP-derived 34-mer peptide; P447-1 is an alanine-substituted 34-mer analog (C455A, C468A); P447-2 is an aspartate-substituted 34-mer analog (C455D, C468D); P447-3 is a proline-substituted 34-mer analog (C455P, I463P, C468P). The optimal inhibitory dose of peptide (1 μg) per mouse was given by the i.p. route. The optimal stimulatory dose of E_2 (0.5 μg per mouse) was given by the i.p. route 1 h after injection of test agent. Twenty-two hours later uteri were dissected, weighed, and compared to uterine weights from no E_2 (negative control) or E_2 alone (positive control) groups.
*Significant inhibition, $P < 0.05$; Wilcoxon sum of ranks test.

Table 2
Antiestrotrophic activity and sequence of AFP-derived small peptides

Test agent	% Inhibition of E_2-stimulated growth of immature mouse uterus	Peptide sequence
Peptide P447	44*	LSEDK LLACG EGAAD IIIGH LCIRH EMTPV NPGV
Peptide P447-4	10	LSEDK LLADG
Peptide P457	0	EGAAD IPIGH
Peptide P467-1	40*	LAIRH EMTPV NPGV
Peptide P471	38*	H EMTPV NPGV
Peptide 472-1	40*	EMTPV NPGV
Peptide P472-2	49*	EMTPV NPG
Peptide P472-3	20	EMTPV NP
Peptide P472-4	5	EMTPV
Peptide P473	0	MTPV NPG
Peptide P474	0	TPV NPGV

P447-4 is an aspartate-substituted 10-mer analog (C455D); P457 is a proline-substituted 10-mer analog (I463P); P467-1 is alanine substituted 14-mer analog (C468A); P471 is a 10-mer analog; P472-1 is a 9-mer analog; P472-2 is an 8-mer analog; P472-3 is a 7-mer analog; P472-4 is a 5-mer analog; P473 is a 7-mer analog missing N-terminal residue of the P472-2; P474 is a 7-mer analog missing two N-terminal residues of P472-2 and addition of C-terminal valine. The optimal inhibitory dose of peptide (1 μg) per mouse was given by the i.p. route. The optimal stimulatory dose of E_2 (0.5 μg per mouse) was given by the i.p. route 1 h after injection of test agent. Twenty-two hours later uteri were dissected, weighed, and compared to no E_2 (negative control) or E_2 alone (positive control). *Significant inhibition, $P < 0.05$; Wilcoxon sum of ranks test.

to identify the active site of peptide P447. Analogs in this category included two 10-mer peptides (P447-4, P457) and one 14-mer peptide (P467-1). The two 10-mer peptides P447-4 and P457 were derived from the amino-terminus and middle of 34-mer peptide P447, while a 14-mer peptide P467-1 was derived from the carboxy-terminus of 34-mer peptide P447. The 14-mer peptide P467-1 exhibited significant antiestrotrophic activity while 10-mer peptides P447-4 and P457 did not (Table 2). Therefore, the antiestrotrophic site was thought to reside within sequence position 467–480.

4. To identify the minimal sequence required for antiestrotrophic activity, truncated forms of the 14-mer 467-1 were synthesized. Analogs in this category included a 10-mer peptide (P471), a 9-mer peptide (P472-1), an 8-mer peptide (P472-2), three 7-mer peptides (P472-3, P473, P474), and a 5-mer peptide (P472-4). All the peptides in this category were derived from the carboxy-terminus of 14-mer peptide P467-1 (Table 2).

Peptide P472-2 is an 8-mer peptide from the C-terminus of the 14-mer, and this small peptide exhibited significant antiestrotrophic activity (Table 2). However, peptides smaller than the 8-mer, P472-3 (7-mer missing the 8-mer C-terminal residue), P473 (7-mer missing N-terminal residue of the 8-mer), P474 (7-mer missing two N-terminal residues of the 8-mer and having an additional C-terminal residue),

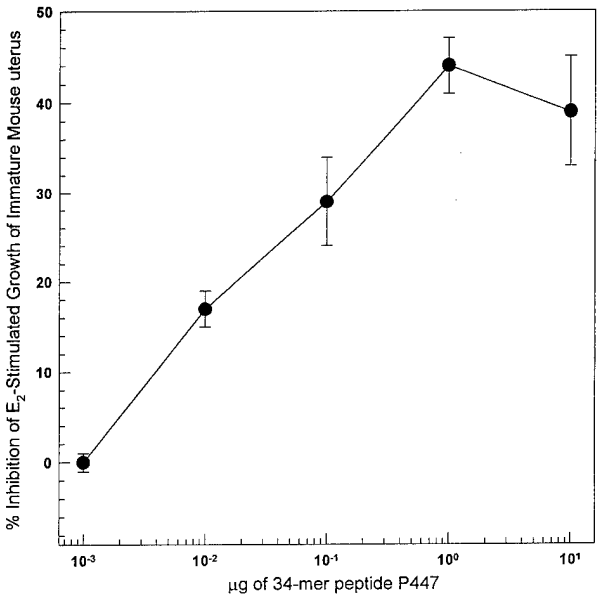

Fig. 2. Dose-dependent antiestrotrophic activity of AFP-derived 34-mer peptide P447. These results demonstrate that AFP-derived 34-mer peptide significantly inhibited estrogen-stimulated growth of immature mouse uterus, and its optimal dose was 1.0 μg per mouse.

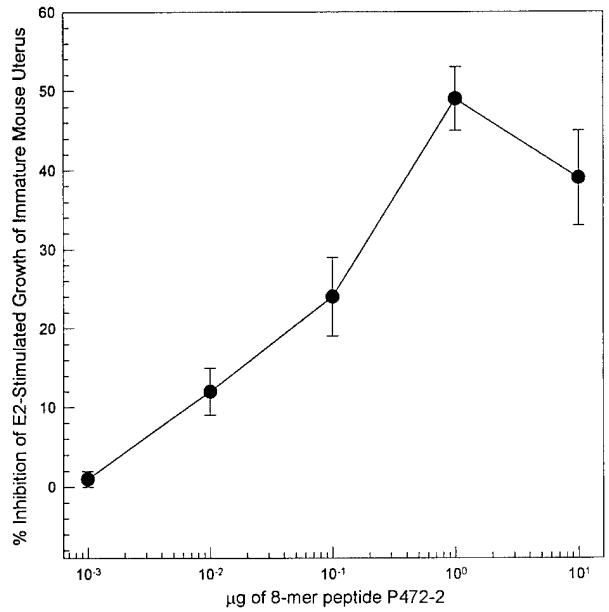

Fig. 3. Dose-dependent antiestrotrophic activity of AFP-derived 8-mer peptide P472-2. These results demonstrate that AFP-derived 8-mer peptide significantly inhibited estrogen-stimulated growth of immature mouse uterus, and its optimal dose was 1.0 μg per mouse.

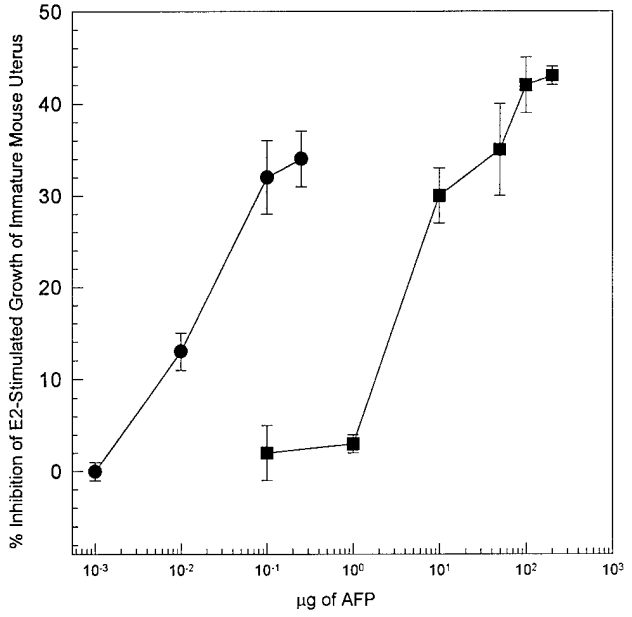

Fig. 4. Dose-dependent antiestrotrophic activity of intact AFP (■) and E_2-transformed AFP (●). These results demonstrate that intact AFP and AFP/E_2 (AFP preincubated with a molar excess estradiol) are antiestrotrophic. Transformed AFP shifts the dose–response curve two logs to the left and mimics the dose–response curve of the peptides.

and P472-1 (5-mer peptide missing three C-terminal residues of the 8-mer) were all without significant inhibitory activity (Table 2). These results indicated that the 8-mer peptide (amino acids 472–479 of AFP) is the minimal sequence from AFP that retains the antiestrotrophic activity of the full-length molecule. A dose–response curve of this peptide is shown in Fig. 3. It is virtually superimposable on the dose–response curve of the 34-mer peptide P447 (Fig. 2) suggesting no loss of activity as a result of truncating the larger peptide.

Interestingly, in comparing the dose–response curves of the two peptides (Figs. 2 and 3) to that of AFP (Fig. 4), it is apparent that the peptides are active at a dose 100-fold lower than AFP. However, as previously reported by our group [4,6,7], preincubation of AFP with a molar excess of estradiol shifts its dose–response curve two logs to the left (Fig. 4) which now mimics the dose–response curve of the peptides. We have speculated that the active site of AFP was more accessible to its receptor in the presence of estradiol, perhaps through an estrogen-mediated conformational change in AFP which alleviated some steric hindrance in placing its active site in apposition to the receptor [11–13]. It would appear

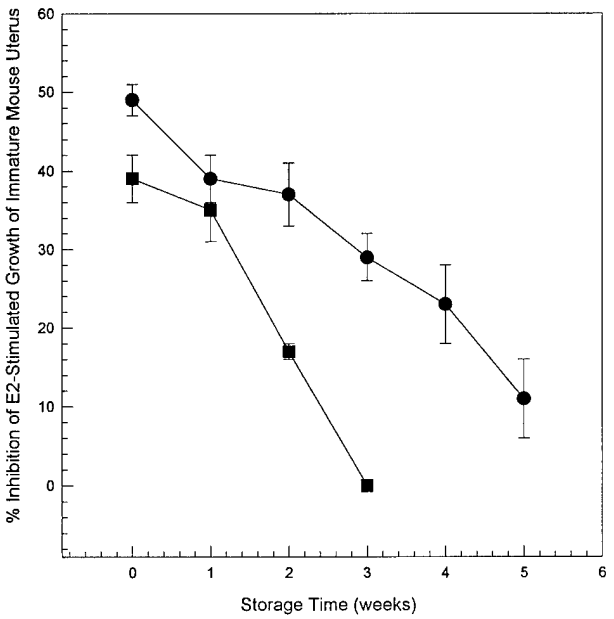

Fig. 5. The antiestrotrophic activity of octapeptide (●) and 34-mer peptide (■) following storage. Peptides were lyophilized and stored at 4°C. These results show that the antiestrotrophic activity of peptides was diminished upon prolonged storage.

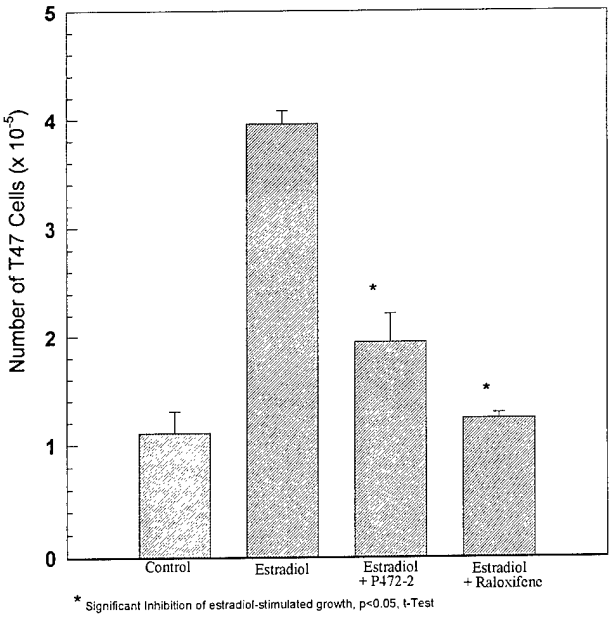

Fig. 6. Growth inhibitory activity of peptide P472-2 against T47D human breast cancer cells growing in culture. The concentration of estradiol (E_2), peptide, and raloxifene (LY156758) were 10 nM, 10 nM, and 100 nM respectively. For peptide and raloxifene these were their maximal inhibitory concentrations. These results show that AFP-derived octapeptide P472-2 inhibits estrogen-stimulated growth of T47D human breast cancer cells in culture.

that such hindrance is not present in either the 34-mer peptide or the 8-mer peptide, and in fact preincubation of these peptides with a molar excess of estradiol did not alter their antiestrotrophic activity (data not shown, [14]). On a molar basis it appears that the estradiol-transformed AFP is more active than the peptides. This may be due to differences in pharmacokinetics and/or active site stability.

It was hoped that the amino acid substitutions in the 34-mer and/or its truncation to the minimal amino acid sequence required for full activity (8-mer, P472-2) would increase the storage stability of these peptides. As shown in Fig. 5, activity of the 8-mer peptide decays more slowly than that of the native 34-mer peptide. None of the substitutions in the 34-mer peptide solved this storability problem. The failure to retain activity during storage is not due to any chemical modification as assessed by mass spectrometry, and is likely attributable to aggregation of these peptides even in their lyophilized state. This aspect of development of these peptides is still under investigation.

The anti-breast cancer activity of the octapeptide was assessed against estradiol-stimulated growth of T47D human breast cancer cells in culture. As shown in Fig. 6, AFP-derived octapeptide inhibited T47D growth by 65% (similar to 34-mer peptide P447, data not shown). Raloxifene (LY 156758) produced almost 100% growth inhibition of these cells in culture.

4. Discussion

The data reported herein demonstrate that an octapeptide, P472-2 (amino acids 472–479 of AFP), possessed the antiestrotrophic activity of full-length AFP. Festin et al. [10,11] had previously shown that this activity was contained in the third domain of AFP (amino acids 386–592), and Mizejewski et al. [14] showed the activity resided in a 34-mer peptide (amino acids 447–480) from domain III of AFP. This 8-mer peptide is a substantially truncated form of the 34-mer and yet retains significant activity. Smaller regions within this 8-mer were without activity. A homology search revealed a five-amino acid sequence (EMTPV, amino acids 472–476 of AFP) that was found in the tumor suppressor protein E-cadherin. This 5-mer peptide was therefore synthesized and tested, and it showed no significant antiestrotrophic activity. The AFP-appropriate amino acids were added to the carboxyl end of this peptide and no significant activity was obtained until the 8-mer (amino acids 472–479 of AFP) was reached. Thus the 8-mer is the minimal sequence required for antiestrotrophic activity, and this appears to be the active site of AFP that imparts this activity to the full-length protein.

Full-length AFP, domain III AFP and the AFP-derived peptides described herein lose activity during storage. Wu et al. [20] reported that AFP tends to form aggregates which may contribute to its loss of activity. Lai et al. reported that peptides are prone to chemical degradation pathways even upon storage in the solid state [21]. Possible chemical modifications of the 8-mer peptide P472-2 might include covalent modifications such as deamination of asparagine, oxidation of methionine, or N-terminal pyroglutamate formation, each of which could be detected by mass spectrometry analysis. Mass spectrometry analysis of the 8-mer peptide P472-2 when freshly prepared, or

after storage in the solid state for over 3 months, indicated the expected molecular weight of 842. This result suggested that the 8-mer peptide P472-2 was free of these chemical modifications and that chemical modification was not the cause of activity loss during storage. In addition to chemical instability, peptides may be prone to physical instability that alters their biological activity [22]. Preliminary studies suggest that the 8-mer peptide and the 34-mer AFP-derived peptides exhibited physical instability, through aggregation. It was noted from gel filtration chromatography profiles that the 34-mer peptide P447 (the parent peptide of P472-2) would readily form dimers, trimers, and large molecular weight aggregates (unpublished observation). This aggregation process of the 34-mer peptide in the lyophilized state resulted in a time-dependent loss of biological activity as assessed by the immature mouse uterine growth bioassay. Preliminary data suggest that the 8-mer, like the 34-mer, forms aggregates during storage in the lyophilized state. Further chemical modifications of the peptide may be necessary to prevent aggregation and concomitant loss of activity during storage.

The mechanism by which AFP and the peptides derived therefrom produce their antiestrotrophic effect is not known. Bennett et al. [4] suggested that sequestering of estrogen by AFP is unlikely because human AFP does not have a high-affinity binding site for estrogen. That the 8-mer retains antiestrotrophic activity further supports this concept because it is highly unlikely that such a small molecule could sequester significant amounts of estrogen. Moreover, the inhibitory mechanism is not like that of raloxifene or tamoxifen in that AFP does not block the binding of estrogen to the estrogen receptor; nor is it like that of letrozole because AFP does not reduce the serum estrogen levels [4]. It is certainly different from cytotoxic chemotherapy, because all evidence has shown AFP to be cytostatic and non-toxic [4]. Although the mechanism of both AFP and AFP-derived peptide is not yet known, when elucidated it is likely to be novel since the more obvious processes described above were not implicated.

AFP is found in the serum of pregnant women, and term pregnancy reduces a woman's future breast cancer risk. Epidemiological studies have been performed to test the hypothesis that AFP in maternal serum (MSAFP) is the agent that reduces risk. Several studies have examined surrogate variables that are known to be associated with elevated serum levels of AFP. It was conjectured that twin pregnancies, in which MSAFP is double that for singleton pregnancies, should generate a much greater reduction of breast cancer risk. Using data from the Cancer and Steroid Hormones Study [23], Jacobson et al. [24] examined the reproductive histories (parity and incidence of twins) of 3918 women who were newly diagnosed with breast cancer and of 4097 control women. As expected, parous women were found to be at lower risk for breast cancer than were nulliparous women. More noteworthy, those who were mothers of twins had a further 40% reduction in risk compared to mothers who had borne only a singleton. Elevated MSAFP is also a characteristic of women who are hypertensive during pregnancy or who are bearing a fetus with an open neural tube defect. Epidemiologic investigations have found that in both of these problem pregnancies, the woman's subsequent breast cancer risk was reduced below that which follows a normal pregnancy [25,26]. Recently, Richardson et al. [27] have reported that the concentration of AFP in cryogenically stored maternal sera was inversely correlated to the risk of breast cancer in these mothers 20–30 years after their pregnancies. These findings support the contention that the action of AFP in maternal serum is preventing appearance of many breast malignancies. In rats, pregnancy reduces the incidence of *N*-nitroso-*N*-methylurea-induced mammary tumor [28]. Sonnenschein et al. [29] have shown that hepatomas secreting AFP resulted in inhibition of estrogen-dependent growth of rat mammary tumors. This suggests that AFP-derived peptides may be developed as agents for breast cancer prevention in women. This speculation is supported by the fact that full-length AFP inhibited the estrogen-stimulated growth of estrogen receptor-positive MCF-7 and T47 human breast cancer xenografts, but did not affect the growth of estrogen receptor-negative MDA-MB-231 and BT20 human breast cancers [4]. It is noteworthy that the AFP preparations which completely inhibited estrogen-stimulated growth of MCF-7 human breast cancer xenografts had 35–45% growth inhibitory activity in the immature mouse uterine growth assay [4]. This suggests that the AFP-derived octapeptide

P472-2, which had antiuterotropic activity in the 40–50% range, will have a potency similar to that of AFP against human breast cancer xenografts. Studies to confirm this speculation are under way.

AFP-derived octapeptide should be more readily adapted for use in the clinic for the treatment of estrogen receptor-positive human breast cancer than full-length AFP. The primary structure of the 8-mer peptide is small relative to full-length AFP (8 vs. 590 amino acids). The peptide is easier to produce. Moreover, since it is a relatively small molecule it will be easier to design analogs. Furthermore, peptides or analogs can be produced in large quantities in forms that are stable on storage. Additionally, peptidomimetic analogs may be produced that would be effective in the treatment of human breast cancer. Since AFP is a natural product, therapeutic agents derived from it may be less toxic than some of the therapeutic agents currently used to treat breast cancer. Furthermore, since almost all breast cancers initially develop as estrogen receptor-positive cells and are estrogen-driven, AFP-derived peptides may be valuable for preventive as well as for therapeutic management of human breast cancers. Thus, further development of this 8-mer peptide appears to be indicated because of its compelling potential to be a novel agent for the management of breast cancer and for the reduction of breast cancer risk in women.

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α-Fetoprotein-derived Anti-Estrogenic Peptide

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Alpha-fetoprotein (AFP) is a major serum protein formed during fetal development. Experimental findings suggest that AFP has anti-estrogenic activity, and the possibility exists that it could be developed as a therapeutic agent to treat existing estrogen-dependent breast cancer or prevent premalignant foci from developing into cancer. Although there is no evidence for AFP binding to estrogen receptor or any other steroid receptors, AFP administration is known to limit the cellular response to estrogen, androgen or thyroid hormone, suggesting AFP may affect cellular processes that are common to the steroid receptor superfamily. Recently, the anti-estrogenic activity of AFP was reported to be localized to a peptide consisting of amino acids 447-480, a 34-mer peptide called p149. Here we report that an 8-mer synthetic peptide (amino acids 472-479) derived from p149 has improved solubility and retains optimal anti-estrogenic activity.

A series of parsings and substitutions of amino acids in the p149 sequence was intended to improve the solubility and enhance structural stability of the peptide. Several peptides related to p149 were generated using solid phase peptide synthesis. Hydrophilic amino acid substitutions in p149 resulted in increased solubility, but substantial substitutions resulted in loss of activity. Shorter peptides, including an 8-mer called peptide 472, retained activity. However, peptides shorter than seven amino acids were inactive. The anti-estrogenic activity of peptide 472 was measured in an assay that determines the inhibition of estrogen-stimulated uterine growth. Peptide 472 exhibited inhibitory activity (40%) comparable to that of p149 (35%) and to that of intact AFP (35-45%). The data suggest that peptide 472 affects the estrogen-receptor mediated response to estrogen in the same manner as does p149 and full length AFP.

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Alpha-Fetoprotein Inhibits Cellular Response to Estrogen, Androgen, Glucocorticoid and Thyroid Hormone. Is it a Superfamily Inhibitor?

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Alpha-fetoprotein (AFP) is a 66.3 Kd, 590 residue single stranded protein that has 39% homology with albumin (Alb), and like Alb is seen as consisting of three structural domains. In Alb these are rigidly oriented by inter-domain disulfide bridges that constrain the molecule to a single conformation. The AFP molecule, however, contains fewer cysteine residues and lacks disulfide bonds between domains II and III, producing a hinge region around which these domains may slue to assume different conformations as influenced by the solute/solvent environment. Adding excess estradiol (E_2) to AFP initiates AFP transformation (observed by difference spectroscopy) to a conformation that is anti-estrotrophic. It inhibits E_2-stimulated growth of rodent uteri and growth of MCF7 breast cancer xenografts in estrogenized immune deficient mice, whereas the same doses of native untransformed AFP are without effect. Androgen-transformed AFP is anti-androgenic, inhibiting androgen-stimulated growth of murine seminal vesicles and of LNCaP prostate cancer xenografts in androgenized immune deficient mice. T_3-transformed AFP antagonizes thyroid hormone action, inhibiting T_3-induced acceleration of tadpole metamorphosis when added to aquarium water. Anti-glucocorticoid activity is also demonstrable. The transformed molecular conformation probably exposes an active site that normally is within a cleft and is inaccessible for reaction with the AFP receptor on cell membranes. In untransformed AFP about 1% of the molecules appears to exist in the transformed state, since full inhibitory effect is seen when the dose is increased by 100 fold. By repeated parsings of the AFP molecule we have generated short peptides that have the same anti-estrotrophic activity shown by transformed full-length AFP, and which are active without the necessity for transformation.

The mechanism by which the active site on AFP antagonizes the cellular responses to superfamily ligands is not yet elucidated. It might involve reaction with a nuclear factor that is common to the transcription complexes of all of the superfamily receptors.

Significance

Within the fetus AFP might have a protective role as an "endocrine limiter". A superfamily ligand concentration rising to inappropriate levels would transform endogenous AFP and thereby attenuate cellular responsiveness to that hormone. AFP could thus serve as a primitive endocrine response regulator prior to the fetus developing its internal endocrine homeostatic mechanisms, and also protect against sporadic high endocrine surges of external (placental or maternal) origin.

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